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# Signal regulatory protein alpha (SIRP $\alpha$ ) regulates the homeostasis of CD103<sup>+</sup>CD11b<sup>+</sup> DCs in the intestinal lamina propria

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Signal regulatory protein alpha (SIRP $\alpha$ /CD172a) is a conserved transmembrane protein thought to play an inhibitory role in immune function by binding the ubiquitous ligand CD47. SIRP $\alpha$  expression has been used to identify dendritic cell subsets across species and here we examined its expression and function on intestinal DCs in mice. Normal mucosa contains four subsets of DCs based on their expression of CD103 and CD11b and three of these express SIRP $\alpha$ . However, loss of SIRP $\alpha$  signaling in mice leads to a selective reduction in the CD103<sup>+</sup>CD11b<sup>+</sup> subset of DCs in the small intestine, colon, and among migratory DCs in the mesenteric lymph node. In parallel, these mice have reduced numbers of T<sub>H</sub>17 cells in steady-state intestinal mucosa, and a defective T<sub>H</sub>17 response to *Citrobacter* infection. Identical results were obtained in CD47KO mice. DC precursors from SIRP $\alpha$  mutant mice had an enhanced ability to generate CD103<sup>+</sup>CD11b<sup>+</sup> DCs *in vivo*, but CD103<sup>+</sup>CD11b<sup>+</sup> DCs from mutant mice were more prone to die by apoptosis. These data show a previously unappreciated and crucial role for SIRP $\alpha$  in the homeostasis of CD103<sup>+</sup>CD11b<sup>+</sup> DCs in the intestine, as well as providing further evidence that this subset of DCs is critical for the development of mucosal T<sub>H</sub>17 responses.

**Keywords:** Dendritic cells • Development • Homeostasis • Intestine • SIRP $\alpha$



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## Introduction

The intestinal immune system is exposed to a wide variety of foreign antigens including dietary constituents, commensal micro-organisms and pathogens. DCs, the professional antigen presenting cells in the gut, must ensure that the correct kind of T cell

is primed so that tolerance or protective immunity is induced appropriately [1, 2]. We and others have shown recently that four distinct subsets of genuine DCs can be identified in the intestine on the basis of CD103 and CD11b expression [2–6], but the contribution of each subset to the different kinds of intestinal immune responses remains largely unknown.

Signal regulatory protein alpha (SIRP $\alpha$ /CD172a) expression is found on the majority of myeloid cells. However, it is expressed differentially by subsets of DCs, being present on CD11b<sup>+</sup> DCs in mice, but not on the DCs with cross-presenting activity

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characterized by expression of CD8 $\alpha$  and CD103 [6–10]. SIRP $\alpha$  is a transmembrane receptor whose cytoplasmic domain contains a tyrosine-based inhibition motif that binds and activates SHP1 and SHP2 phosphatases [9, 11, 12]. The ligand for SIRP $\alpha$  is the ubiquitously expressed CD47 and this interaction is generally believed to have inhibitory effects on immune function, having been implicated in the pathogenesis of a number of models of autoimmunity including experimental autoimmune encephalomyelitis, contact hypersensitivity, and collagen-induced arthritis [13–16].

The SIRP $\alpha$ -CD47 axis has also been implicated in regulating immunity in the gut, although the exact effects and basis for this regulation remain unclear. Thus although CD47KO mice have reduced susceptibility to experimental colitis [17] and decreased intestinal IgA production, they show normal tolerance when administered protein antigens orally [18].

SIRP $\alpha$  mutant mice [19], which have a truncated cytoplasmic domain of the protein and hence cannot signal intracellularly, have a reduction in the proportion of flagellin inducible IL-17- and IFN- $\gamma$ -producing T cells in the intestinal lamina propria (LP) [20]. Although some of these effects have been linked to abnormalities in the SIRP $\alpha^+$  (CD11b $^+$ ) subset of “DCs” [18, 20], their interpretation is clouded by the fact that these cells were identified only on the basis of expression of CD11c and MHCII. Furthermore it was assumed that CD11b and CD103 defined mutually exclusive subsets of intestinal DCs as they do in other tissues [21]. Recently, we and others have shown that most CD11b $^+$ CD11c $^+$ MHCII $^+$  cells in the intestinal mucosa are resident macrophages rather than the migratory DCs that are needed to drive naïve T-cell priming. In addition, a substantial population of intestinal DCs express both CD103 and CD11b [4, 22–24]. Here, we have exploited more rigorous identification strategies to reexamine how the SIRP $\alpha$ -CD47 axis regulates intestinal immunity. We show that although SIRP $\alpha$  is expressed by macrophages and three distinct populations of DCs in the gut; the loss of CD47 or SIRP $\alpha$  signaling leads to a selective decrease in CD103 $^+$ CD11b $^+$  DCs, together with a decrease in the generation of intestinal T<sub>H</sub>17 cells. The loss of CD103 $^+$ CD11b $^+$  DCs in SIRP $\alpha$  mutant mice appears to reflect enhanced susceptibility of these cells to die by apoptosis rather than defective generation from DC progenitors.

## Results

### Loss of SIRP $\alpha$ -CD47 signaling results in a specific reduction in intestinal CD103 $^+$ CD11b $^+$ DCs

We first investigated exactly which small intestine lamina propria (SI LP) DC subsets expressed SIRP $\alpha$ , using our recently established gating strategy in which bona fide DCs among mucosal mononuclear phagocytes (MPs) are identified as CD11c $^+$ MHCII $^+$ CD64 $^-$ F4/80 $^-$  [3, 4, 22, 30]. On this basis, three SIRP $\alpha$ -expressing subsets of DCs could be observed: CD103 $^+$ CD11b $^+$ , CD103 $^-$ CD11b $^+$ , and CD103 $^-$ CD11b $^-$  (Fig. 1A). The SIRP $\alpha^+$  DC population contained both CD103 $^+$ CD11b $^-$  and CD103 $^-$ CD11b $^-$

subsets, with all CD103 $^+$ CD11b $^-$  DCs failing to express SIRP $\alpha$ , whereas the CD103 $^-$ CD11b $^-$  population was heterogeneous for SIRP $\alpha$  expression. SIRP $\alpha$  expression was mutually exclusive to that of CD8 $\alpha$ , which was found on all CD103 $^+$ CD11b $^-$  DCs and on some CD103 $^-$ CD11b $^-$  DCs, but not on the CD11b $^+$  subsets (Fig. 1A). As expected [4, 22], all CD11c $^+$ MHCII $^+$ CD64 $^+$ F4/80 $^+$  resident macrophages also expressed SIRP $\alpha$  (Fig. 1A). Similar patterns of staining were observed among the mononuclear phagocytes in the colonic LP (Fig. 1B) and among migratory DC (CD11c $^+$ MHCII $^{\text{hi}}$ ) in the mesenteric lymph nodes (Fig. 1C).

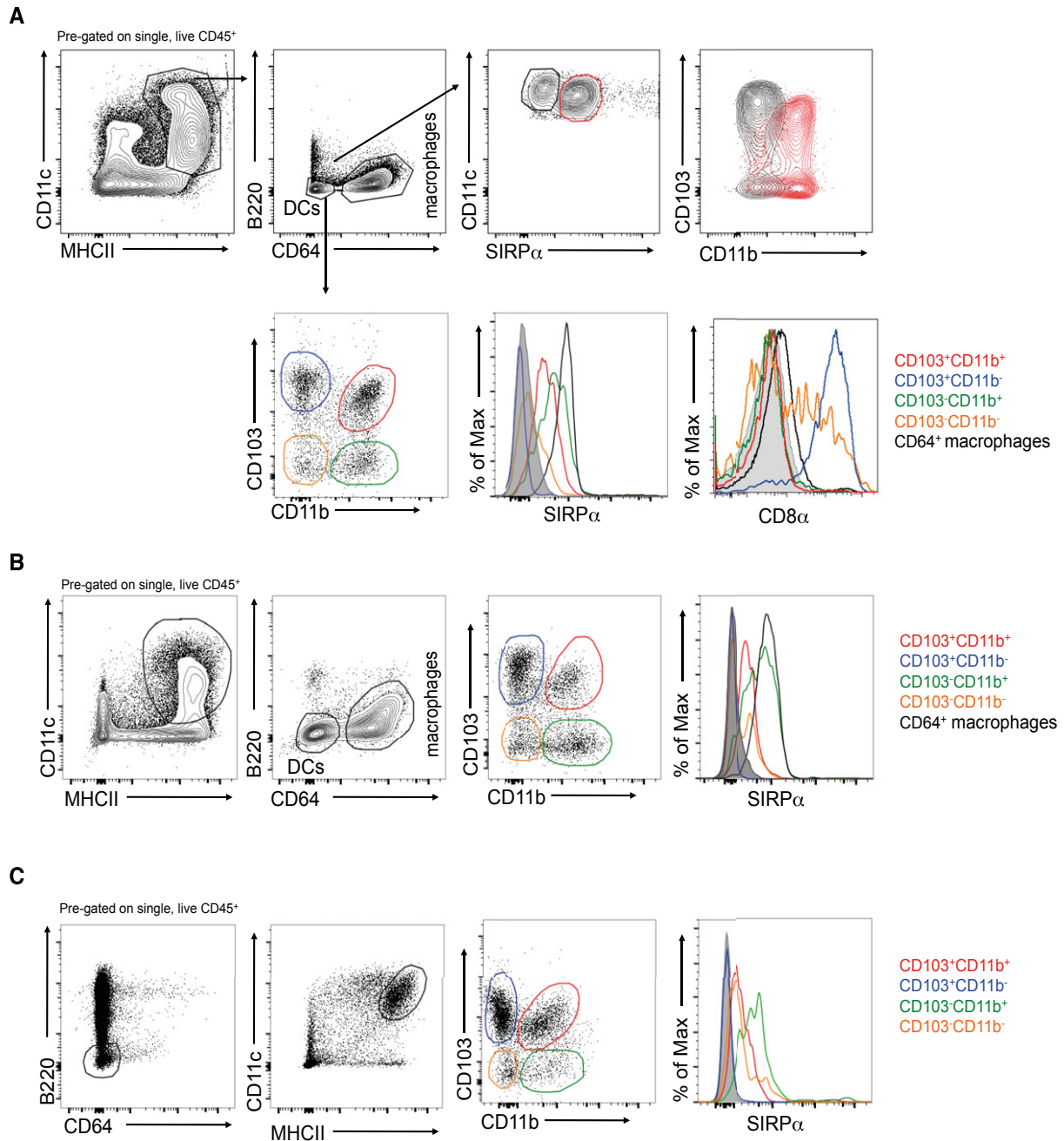
Next, we investigated whether SIRP $\alpha$  played a functional role in intestinal DCs, using mice with a truncated cytoplasmic domain of SIRP $\alpha$  (SIRP $\alpha$  mt) that cannot signal intracellularly [19]. These mice had a selective reduction in the proportion and absolute number of CD103 $^+$ CD11b $^+$  DCs in the SI (Fig. 2A), colon (Supporting Information Fig. 1), and among migratory CD11c $^+$ MHCII $^{\text{hi}}$  DCs in the MLNs (Fig. 2B). Although the absolute numbers of all migratory DC subsets were reduced in the MLNs of SIRP $\alpha$  mt mice, this reflected a global reduction in cellularity and only the CD103 $^+$ CD11b $^+$  DCs showed a proportional defect in mutant MLNs (Fig. 2C). The other SIRP $\alpha$  expressing intestinal MPs including macrophages, were unaffected by the loss of SIRP $\alpha$  signaling in either the SI or colon (Fig. 2D and Supporting Information Fig. 1C).

### CD47KO mice phenocopy the DC defect in SIRP $\alpha$ mt mice

CD47KO mice had a selective and equivalent reduction in CD103 $^+$ CD11b $^+$  DCs in both the SI LP and migratory compartment of the MLNs (Fig. 3A and B), as well as normal proportions and numbers of mucosal macrophages (Fig. 3C).

### Reduction in CD103 $^+$ CD11b $^+$ DCs correlates with a selective defect in intestinal T<sub>H</sub>17 cells

As CD103 $^+$ CD11b $^+$  DCs have recently been implicated in the homeostasis of mucosal T<sub>H</sub>17 cells [5, 6, 13, 26–29], we next examined the CD4 $^+$  T-cell compartment in the small intestinal LP of steady-state SIRP $\alpha$  mt and CD47KO animals. Both SIRP $\alpha$  mt and CD47KO mice showed an approximately 50% reduction in IL-17-producing T<sub>H</sub>17 cells compared with WT LP, whereas the numbers of FoxP3 $^+$  Treg and IFN- $\gamma^+$  T<sub>H</sub>1 cells were unaffected (Fig. 4A and B, and Supporting Information Fig. 2A). In addition, SI LP CD4 $^+$  T cells FACS-purified from SIRP $\alpha$  mt mice had a trend toward reduced il22 mRNA expression (Supporting Information Fig. 2B). During infection by the intestinal pathogen *Citrobacter rodentium*, SIRP $\alpha$  mt mice also showed defective induction of T<sub>H</sub>17 cells in the MLNs, as well as a trend toward reduced proportions of CD4 $^+$  T cells producing IL-22 in the colonic LP, where there was a ~40% reduction compared with the levels in WT colon (Fig. 4C and D, and Supporting Information Fig. 2C). These changes in T-cell differentiation were associated with

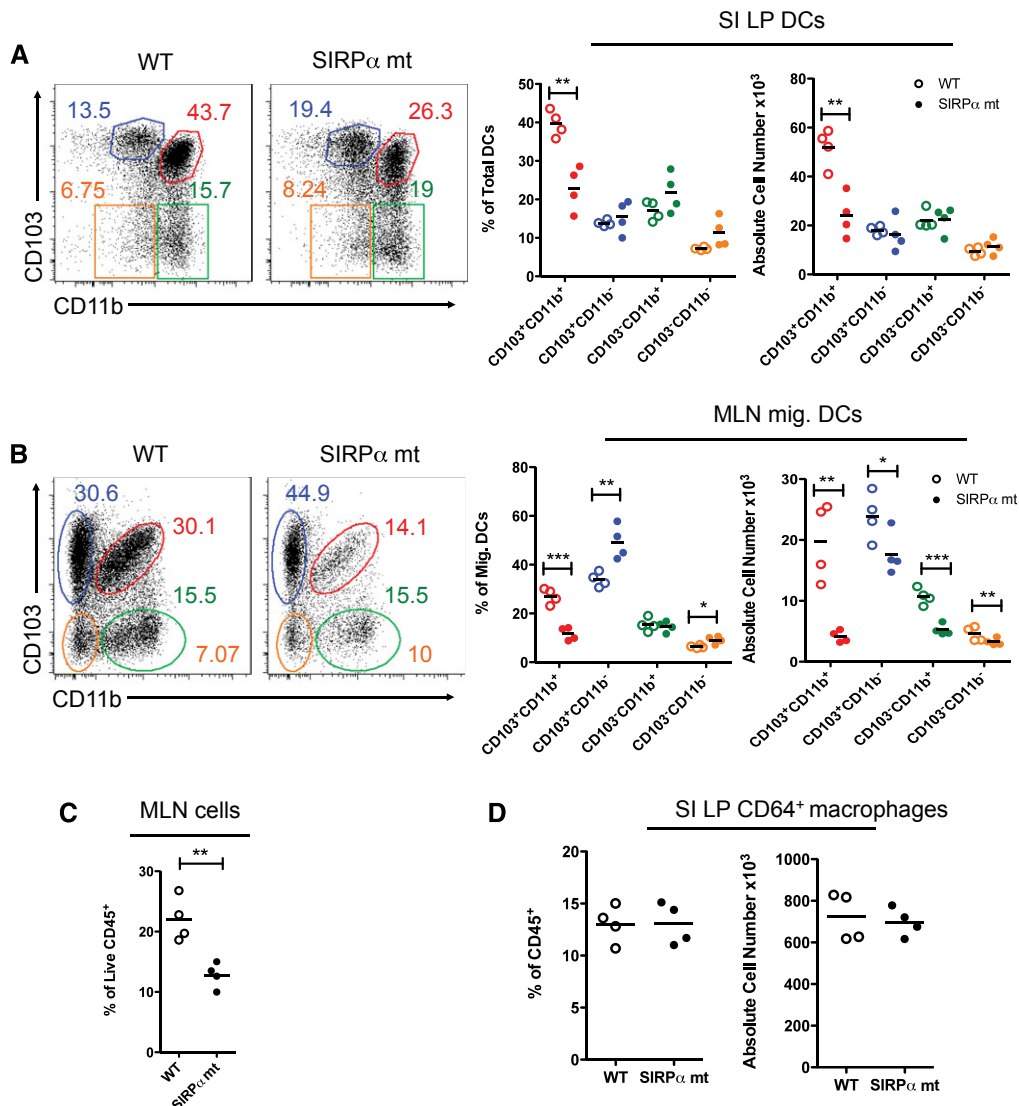


**Figure 1.** SIRP $\alpha$  expression on intestinal mononuclear phagocytes. (A) Mononuclear phagocytes were identified among live single CD45<sup>+</sup> cells from enzymatically digested small intestinal lamina propria as CD11c<sup>+</sup>MHCII<sup>+</sup>. Contaminating B cells were excluded on the basis of B220 expression and DCs and macrophages were identified as CD64<sup>-</sup> or CD64<sup>+</sup>, respectively. Top panels: Expression of CD103 and CD11b by CD11c<sup>+</sup>SIRP $\alpha$ <sup>-</sup> (black) and CD11c<sup>+</sup>SIRP $\alpha$ <sup>+</sup> (red) DCs. Bottom panels: Expression of SIRP $\alpha$  and CD8 $\alpha$  by DC subsets and CD64<sup>+</sup> macrophages (isotype controls shown in gray). (B) Expression of SIRP $\alpha$  by mononuclear phagocytes in the colonic LP gated as described in (A). (C) Expression of SIRP $\alpha$  by CD103<sup>-</sup>CD11b<sup>+</sup> subsets of migratory (CD11c<sup>+</sup>MHCII<sup>hi</sup>) DCs in the mesenteric lymph node. (A–C) Plots are from one experiment representative of at least six independent experiments, with  $n = 3$ –4 mice/experiment.

delayed clearance of the pathogen (Fig. 4E). Importantly, these differences were not due to impaired IL-22 production by type 3 innate lymphoid cells (ILC3s) (Supporting Information Fig. 2D).

As we have recently shown that CD103<sup>-</sup>CD11b<sup>+</sup> DCs from the intestine are the main inducers of T<sub>H</sub>17 differentiation in

vitro [3, 30], we assessed whether a functional defect in this population could account for the impaired T<sub>H</sub>17 priming in SIRP $\alpha$  mt mice, despite the normal numbers of this subset. However, CD103<sup>-</sup>CD11b<sup>+</sup> DCs from the SI LP of SIRP $\alpha$  mt mice were equally capable of inducing T<sub>H</sub>17 responses as their WT counterparts following ex vivo coculture with naïve CD4<sup>+</sup> OTII T cells



**Figure 2.** SIRP $\alpha$  signaling controls the homeostasis of CD103<sup>+</sup>CD11b<sup>+</sup> DCs in vivo. (A) Proportions and absolute numbers of CD103/CD11b-based subsets among live CD45<sup>+</sup>CD11c<sup>+</sup>MHCII<sup>+</sup>CD64<sup>+</sup>B220<sup>+</sup> DCs from small intestinal LP of SIRP $\alpha$  mutant (mt) (filled circles) and WT (empty circles) mice. (B) Proportions and absolute numbers of CD103/CD11b-based subsets among CD11c<sup>+</sup>MHCII<sup>hi</sup> migratory DCs in the MLNs of SIRP $\alpha$  mt and WT mice. (C) Frequency of live total CD45<sup>+</sup> cells in the MLNs of SIRP $\alpha$  mt and WT mice. (D) Proportions and numbers of CD64<sup>+</sup> macrophages among live CD45<sup>+</sup>CD11c<sup>+</sup>MHCII<sup>+</sup> cells in small intestinal LP of SIRP $\alpha$  mt and WT mice. Data are from one experiment representative of at least five independent experiments, with  $n = 3/4$  per experiment. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.005$ ; Student's *t*-test.

(Fig. 4F). The reduced T<sub>H</sub>17 cell generation in vivo was also not due to an intrinsic defect in SIRP $\alpha$  mt T cells, as naïve CD4<sup>+</sup> T cells from SIRP $\alpha$  mt MLNs could be polarized in vitro to express ROR $\gamma$ t and IL17a at levels equivalent to WT MLN CD4<sup>+</sup> T cells (Fig. 4G).

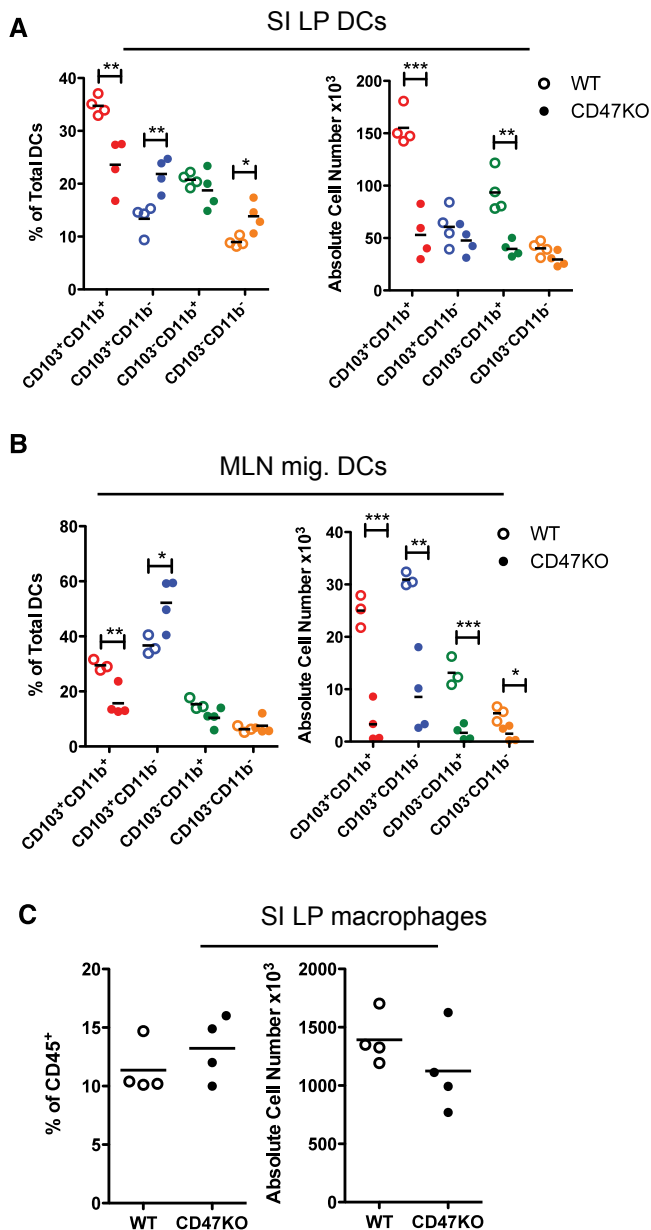
In contrast to this defect in T<sub>H</sub>17 cell generation, regulatory T cell dependent mechanisms appeared to be normal in the absence of SIRP $\alpha$  signaling. Thus there were normal numbers and proportions of FoxP3<sup>+</sup> Treg cells in the SIRP $\alpha$  mt SI and colon (Fig. 4A and B, and Supporting Information Fig. 2A and E), although a slight reduction was observed in the MLNs (Supporting Information Fig. 2F). In addition these mice developed tolerance of systemic delayed type hypersensitivity responses nor-

mally when fed OVA before parenteral challenge with antigen in CFA (Supporting Information Fig. 2G).

### Development of CD103<sup>+</sup>CD11b<sup>+</sup> DC from precursors is enhanced by the loss of SIRP $\alpha$ -CD47 signaling

As CD103<sup>+</sup>CD11b<sup>+</sup> intestinal DCs are the progeny of DC-committed progenitors that express SIRP $\alpha$  [23, 25, 30], we explored whether defective SIRP $\alpha$  signaling might affect the generation of these DCs. Normal numbers of pre-DCs were present in the BM and blood of SIRP $\alpha$  mt mice [31, 32] (Fig. 5A). To study their ability to generate intestinal DCs in vivo, CD45.1<sup>+</sup> WT





**Figure 3.** CD47KO mice phenocopy the intestinal DC defect in SIRP $\alpha$  mt mice. (A) Proportions and absolute numbers of CD103/CD11b-based subsets among live CD45<sup>+</sup>CD11c<sup>+</sup>MHCII<sup>+</sup>CD64<sup>+</sup>B220<sup>+</sup> DCs from small intestinal LP of CD47KO (filled circles) and WT (empty circles) mice. (B) Proportions and absolute numbers of CD103/CD11b-based subsets among CD11c<sup>+</sup>MHCII<sup>hi</sup> migratory DCs in the MLNs of CD47KO and WT mice. (C) Proportions and numbers of CD64<sup>+</sup> macrophages among live CD45<sup>+</sup>CD11c<sup>+</sup>MHCII<sup>+</sup> cells in small intestinal LP of SIRP $\alpha$  mt and WT mice. Data are from one experiment representative of at least three independent experiments, with  $n = 3/4$  per experiment. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.005$ ; Student's *t*-test.

and CD45.2<sup>+</sup> SIRP $\alpha$  mt pre-DCs were transferred in a 50:50 ratio into CD45.1<sup>+</sup>/CD45.2<sup>+</sup> WT recipient mice (Fig. 5B). The mature progeny were then identified in the SI LP 5 days after transfer (Fig. 5C), a time we had found optimal for the development of DCs in the gut in this system (data not shown). Unexpectedly, SIRP $\alpha$  mt pre-DCs appeared to be more effective at generating

CD103<sup>+</sup>CD11b<sup>+</sup> DCs in LP than their WT counterparts, as well as being somewhat better at generating SIRP $\alpha$ <sup>+</sup>CD103<sup>+</sup>CD11b<sup>+</sup> DCs (Fig. 5D). SIRP $\alpha$  mt and WT pre-DCs had equal abilities to generate the two CD103<sup>+</sup> DC populations, both of which express SIRP $\alpha$  (Fig. 5D).

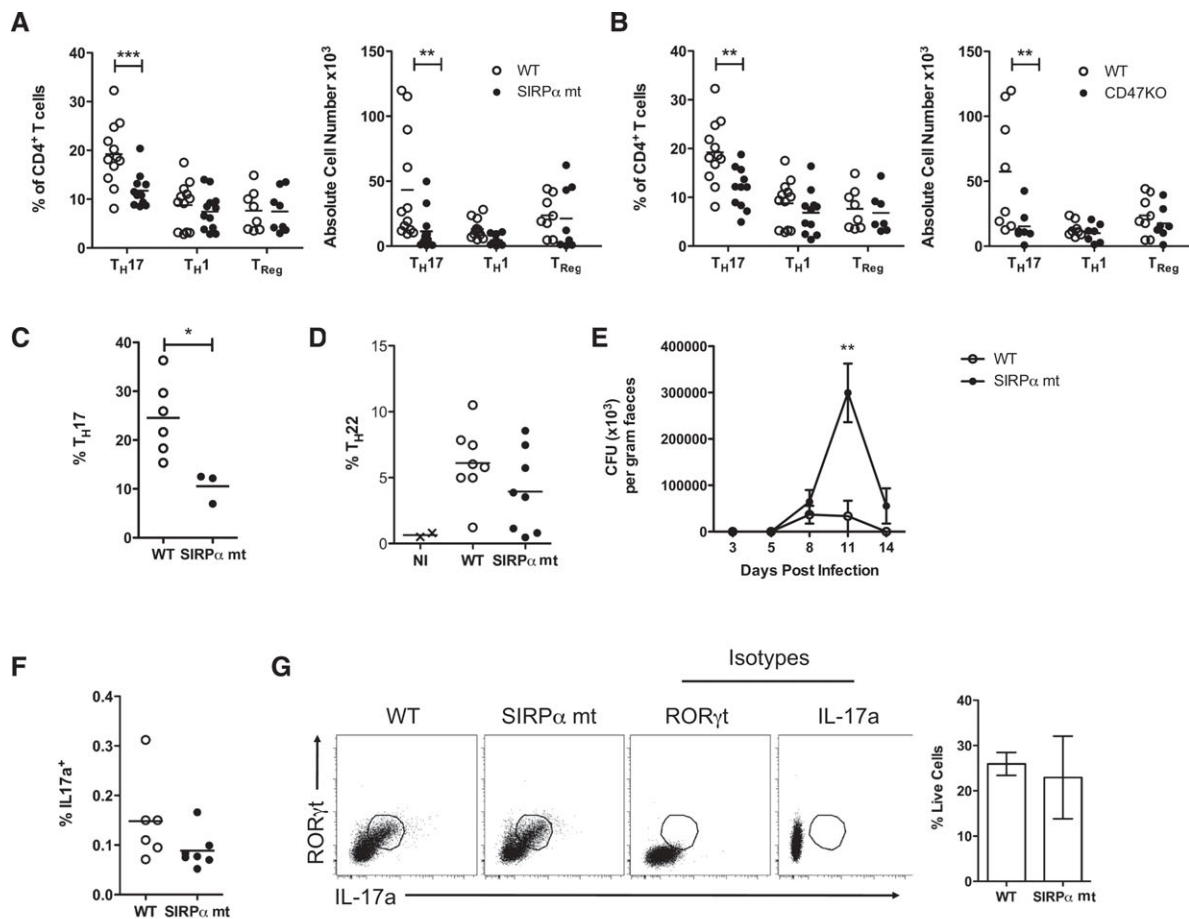
### Increased apoptosis of CD103<sup>+</sup>CD11b<sup>+</sup> DCs in the absence of a functional SIRP $\alpha$ signal

The selective advantage of SIRP $\alpha$  mt pre-DCs in generating CD103<sup>+</sup>CD11b<sup>+</sup> DCs, despite the marked deficiency in this subset seen in SIRP $\alpha$  mice, suggested that these DCs might be compromised by the lack of SIRP $\alpha$  signaling later in their life. To explore this, we compared the apoptosis of intestinal DC subsets in SIRP $\alpha$  mt and WT mice. Annexin V staining showed that migratory CD103<sup>+</sup>CD11b<sup>+</sup> DCs were more prone to apoptosis in SIRP $\alpha$  than in WT MLNs. In contrast, no differences in Annexin V staining were noted among the other DC populations (Fig. 6). Thus the SIRP $\alpha$ /CD47 axis appears to be important for promoting the survival of intestinal CD103<sup>+</sup>CD11b<sup>+</sup> DCs.

## Discussion

Here, we have exploited recent advances in characterizing intestinal DCs and their subsets to explore the significance of SIRP $\alpha$  expression on these cells. We demonstrate that three of the four subsets of bona fide DCs, that we and others have identified in the mouse intestine [3–5, 30] express SIRP $\alpha$ , these being CD103<sup>+</sup>CD11b<sup>+</sup>, CD103<sup>+</sup>CD11b<sup>+</sup>, and CD103<sup>+</sup>CD11b<sup>+</sup>. The remaining CD103<sup>+</sup>CD11b<sup>+</sup> DCs are uniformly SIRP $\alpha$ <sup>+</sup> and comprise the CD8 $\alpha$ <sup>+</sup>DNGR-1<sup>+</sup>XCR1<sup>+</sup> population responsible for cross-presentation [33]. Some CD103<sup>+</sup>CD11b<sup>+</sup> DCs also fail to express SIRP $\alpha$ , consistent with previous findings that this subset is phenotypically heterogeneous and its functions remain to be elucidated [3].

Despite its widespread expression, loss of SIRP $\alpha$  signaling in SIRP $\alpha$  mt mice caused a selective reduction in CD103<sup>+</sup>CD11b<sup>+</sup> DCs in the LP of the entire intestinal tract and among migratory DCs in MLNs. The other subsets of SIRP $\alpha$ <sup>+</sup> DC were unaffected, as were CD64<sup>+</sup> macrophages, which are uniformly SIRP $\alpha$ <sup>+</sup>. Identical results were obtained in mice lacking the ligand for SIRP $\alpha$ , CD47, extending a previous study that found reduced CD103<sup>+</sup>CD11b<sup>+</sup> LP DCs in CD47KO mice, but in which the other DC populations were not examined [18]. Other groups also found reduced numbers of CD11b<sup>+</sup> “DCs” in the LP of SIRP $\alpha$  mt and CD47KO mice, but these were reported to lack CD103 and the cells analyzed were total CD11c<sup>+</sup>CD11b<sup>+</sup> MPs [17, 20]. As we show here, this population is highly heterogeneous, containing macrophages, CD103<sup>+</sup>CD11b<sup>+</sup> DCs and CD103<sup>+</sup>CD11b<sup>+</sup> DCs, which can only be distinguished by multiparameter analysis. As CD103<sup>+</sup>CD11b<sup>+</sup> DCs are a relatively minor part of this overall population, they could easily have been overlooked in earlier studies.

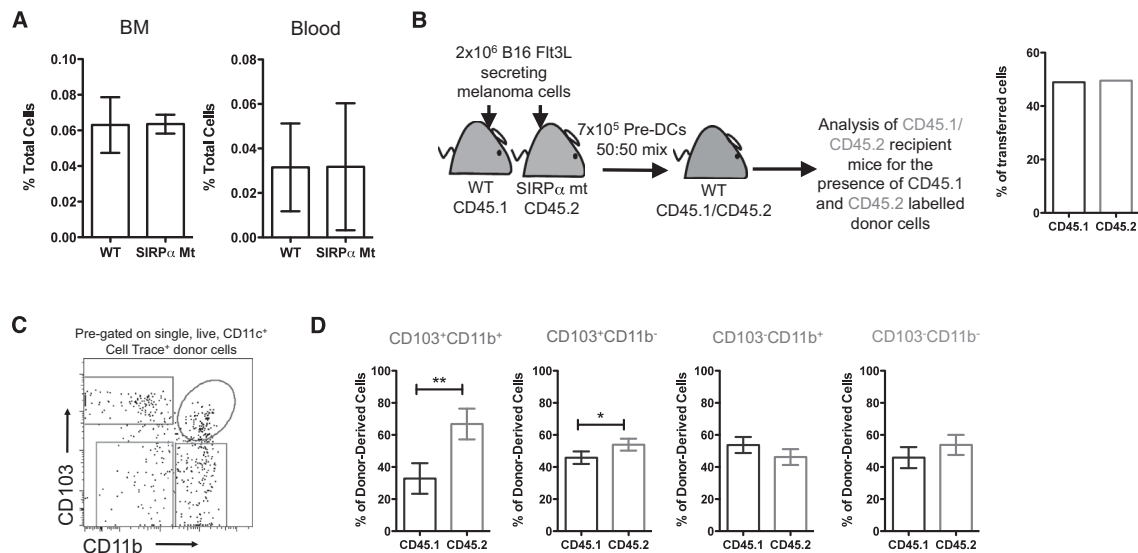


**Figure 4.** Defects in CD103<sup>+</sup>CD11b<sup>+</sup> intestinal DCs correlate with reduced TH17 cells in LP. (A, B) Proportions and absolute numbers of cells staining intracellularly for IL-17a, FoxP3, and IFN- $\gamma$  among total live CD4<sup>+</sup> T cells in the small intestinal LP of SIRP $\alpha$  mt mice (A) and CD47KO mice (B) (filled circles), together with WT controls (empty circles). (A and B) Data are pooled from 2–3 independent experiments, with  $n = 4$  per experiment. (C) Mice were infected orally with  $1 \times 10^9$  CFU of *C. rodentium* and the numbers of IL-17a producing CD4<sup>+</sup> T cells in MLNs assessed on day 8 of infection by intracellular cytokine staining. Results show proportion of TH17 cells as a percentage of total CD4<sup>+</sup> T cells in the MLN of SIRP $\alpha$  mt and WT mice. Data are from one experiment representative of two independent experiments, with  $n = 3$ –6 mice per experiment. (D) IL-22 producing CD4<sup>+</sup> T cells in the colonic LP of SIRP $\alpha$  mt (filled circles) and WT (empty circles) mice 8 days after infection with *C. rodentium* as assessed by intracellular cytokine staining. Results show the proportions of IL-22 producing cells as a percentage of total CD4<sup>+</sup> T cells. Data are from a single experiment with  $n = 2$ –8 mice/group. NI (x) represents noninfected WT controls. (E) Course of infection with *C. rodentium* in WT and SIRP $\alpha$  mt mice. The data are shown as mean  $\pm$  SD (CFU  $\times 10^3$ /g feces) from ten mice/group and are from one experiment representative of two experiments. (F) CD103<sup>+</sup>CD11b<sup>+</sup> DCs ( $3 \times 10^4$ ) were FACS-purified from the SI LP of WT and SIRP $\alpha$  mt mice, pulsed with 2 mg/mL OVA and cocultured with FACS-purified naïve (CD62L<sup>+</sup>CD25<sup>−</sup>) CD4<sup>+</sup> OVA-specific OTII transgenic T cells for 4 days before being assessed for IL-17a production by intracellular cytokine staining. Data are pooled from three independent experiments. (G) FACS-purified naïve CD62L<sup>+</sup>CD25<sup>−</sup> CD4<sup>+</sup> T cells from the MLN of SIRP $\alpha$  mt or WT mice were cultured for 4 days on plates coated with anti-CD3 and anti-CD28, together with anti-IFN- $\gamma$ , anti-IL-4, anti-IL-2, IL-6, IL-23, IL-1 $\beta$ , and TGF- $\beta$ . ROR $\gamma$ t and IL-17a expression were then assessed by intracellular staining. Data are shown as means  $\pm$  SD pooled from two independent experiments, with  $n = 6$ –7 per group. \* $p < 0.05$ , \*\* $p < 0.01$ ; Student's  $t$ -test

As has been reported previously [13, 34–36], the reduction in CD103<sup>+</sup>CD11b<sup>+</sup> DCs which we observed in the intestine of SIRP $\alpha$  mt and CD47KO mice was accompanied by a defect in CD11b expressing DCs in other tissues such as the spleen. However, the phenotypically identical CD103<sup>+</sup>CD11b<sup>+</sup> DC subset in the intestine was not affected by the SIRP $\alpha$  mutation. CD103 expression on CD11b<sup>+</sup> (SIRP $\alpha$ <sup>+</sup>) DCs is unique to the intestine and appears to reflect tissue specific "conditioning" by that environment [2]. Similarly concordant defects in CD11b<sup>+</sup> splenic DCs and in the CD103<sup>+</sup>CD11b<sup>+</sup> subset in LP are also present in mice with IRF4 and Notch2 deficiency targeted to CD11c<sup>+</sup> cells, but the effects

on CD103<sup>+</sup>CD11b<sup>+</sup> DCs in the intestine of these mice remain to be investigated [5, 6, 26, 37]. Together, our results could suggest a model in which mucosal CD103<sup>+</sup>CD11b<sup>+</sup> DCs are less mature than either the CD103<sup>+</sup>CD11b<sup>+</sup> DCs in intestine or CD11b<sup>+</sup> DC in the spleen, and that SIRP $\alpha$  signaling is essential for their full differentiation. We are currently examining this idea in more detail.

The loss of CD103<sup>+</sup>CD11b<sup>+</sup> intestinal DCs in SIRP $\alpha$  mt and CD47KO mice was accompanied by a selective reduction in IL-17/IL-22 producing CD4<sup>+</sup> T cells in the steady-state intestinal LP, whereas TH1 cells and FoxP3<sup>+</sup> Treg cells were unaffected. Similar defects in TH17/22 generation were found during



**Figure 5.** Loss of functional SIRP $\alpha$  signal confers a selective advantage in the generation of CD103<sup>+</sup>CD11b<sup>+</sup> DCs from pre-DCs. (A) Proportions of Lin<sup>-</sup>CD11c<sup>int</sup>SIRP $\alpha$ <sup>int</sup>CD135<sup>+</sup> pre-DCs in the BM and blood of WT and SIRP $\alpha$  mt mice. Data are shown as % of total cells  $\pm$  1 SD and are from one experiment representative of 2–5 independent experiments with  $n = 3–4$  per group per experiment. (B) Lin<sup>-</sup>CD11c<sup>int</sup>SIRP $\alpha$ <sup>int</sup>CD135<sup>+</sup> pre-DCs were FACS sorted from the BM of CD45.1<sup>+</sup> WT and CD45.2<sup>+</sup> SIRP $\alpha$  mt mice ten days after subcutaneous injection of  $2 \times 10^6$  Flt3L secreting B16 cells, labeled with CellTrace violet dye, mixed in a 50:50 ratio and  $7 \times 10^5$  cells were transferred i.v. into resting CD45.1<sup>+</sup>/CD45.2<sup>+</sup> WT recipients. (C) Five days later, CellTrace<sup>+</sup> total donor cells were identified in the SI LP of recipient mice and examined for CD103 and CD11b expression. (D) Donor-derived DCs were then examined for CD45.1 and CD45.2 expression to assess their origin from WT versus SIRP $\alpha$  mt precursors. The data are shown as the mean  $\pm$  SD ( $n = 4$ ) and are pooled from two independent experiments. \* $p < 0.05$ , \*\* $p < 0.01$ , Student's  $t$ -test.

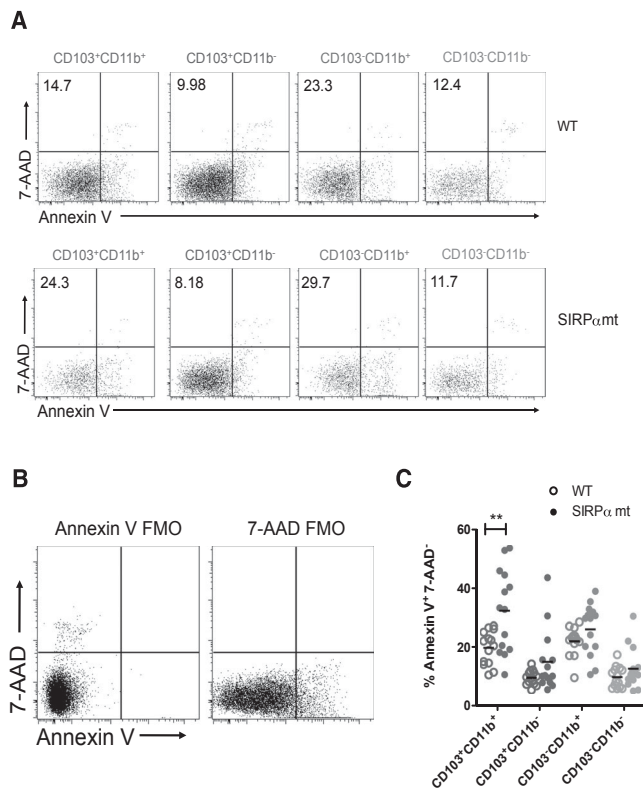
infection with *C. rodentium* in SIRP $\alpha$  mt mice, which also showed delayed clearance of the organism. Protective immunity in this infection requires IL-22 and IL-17, produced by different cells in two distinct phases. Early in infection, these cytokines are derived from ILC3s, whereas CD4<sup>+</sup> T cells are needed for the later stage in which the pathogen is cleared [38]. The role of CD103<sup>+</sup>CD11b<sup>+</sup> DCs in this infection has been controversial, as although one report suggested that they were required to drive the early IL-22 production by ILC3s [37], others found that CD103<sup>+</sup>CD11b<sup>+</sup> DCs were not required for clearance of the organism [27]. Here, we found that the delayed clearance in SIRP $\alpha$  mt mice only became apparent at the later stages of infection and this correlated with fewer T<sub>H</sub>17 and T<sub>H</sub>22 cells in the MLNs and colonic LP. In contrast, we could not find any defect in IL-22 production by ILC3 in SIRP $\alpha$  mt mice. For these reasons we conclude that the enhanced susceptibility of these mice to *C. rodentium* infection reflects defective priming of adaptive effector T cells by CD103<sup>+</sup>CD11b<sup>+</sup> DCs rather than an effect on ILC3s.

Our findings of reduced generation of intestinal T<sub>H</sub>17 cells in SIRP $\alpha$  mt mice are consistent with other disease models in these mice, including EAE, contact hypersensitivity and collagen-induced arthritis [13, 14, 16]. Recent studies in other mouse models have also confirmed a specific link between reduced numbers of CD103<sup>+</sup>CD11b<sup>+</sup> DCs and fewer T<sub>H</sub>17 cells in the gut, perhaps reflecting reduced production of polarizing cytokines such as IL-6 or IL-23 [5, 6, 26, 27, 37]. However, the exact mechanisms underlying the connection remain to be elucidated and it should be noted that our recent studies show that CD103<sup>-</sup>CD11b<sup>+</sup> DCs are the most effective inducers of T<sub>H</sub>17 cell differentiation when

DC subsets from intestinal LP or lymph are assessed using naïve antigen-specific CD4<sup>+</sup> T cells in vitro [3, 30]. The explanation for these apparently discordant results is unknown, but could indicate that the CD103<sup>+</sup> and CD103<sup>-</sup> subsets of CD11b<sup>+</sup> DCs may represent different stages in the same developmental pathway, or they may need to interact together in vivo to generate T<sub>H</sub>17 cells. Alternatively the subsets may play separate roles in the induction and subsequent maintenance of T<sub>H</sub>17 cells in vivo. This latter idea could be consistent with work suggesting that the effects of CD103<sup>+</sup>CD11b<sup>+</sup> DCs on the homeostasis of T<sub>H</sub>17 cells in the intestine may not require presentation of cognate antigen [27]. Furthermore, recent studies show that the induction of T<sub>H</sub>17 cells in the intestine by Segmented Filamentous Bacteria involves two processes, one of which requires uptake via secondary lymphoid organs and presentation of specific antigen by DCs; a further population of segmented filamentous bacteria-dependent T<sub>H</sub>17 cells is independent of these events [39, 40]. More work is needed to define better the link between different DC subsets and T<sub>H</sub>17 cell homeostasis in the intestine.

The loss of SIRP $\alpha$  signaling did not affect the numbers of FoxP3<sup>+</sup> Treg in the steady-state LP, and SIRP $\alpha$  mt mice developed systemic tolerance normally after feeding protein, a phenomenon that is dependent on Treg cells [1]. These results are consistent with previous studies showing normal oral tolerance in CD47KO mice [18] and with other recent work showing normal numbers of FoxP3<sup>+</sup> Treg cells when CD103<sup>+</sup>CD11b<sup>+</sup> DCs are lacking [2, 27]. Thus original assumptions that intestinal CD103<sup>+</sup> DCs were intrinsically tolerogenic need to be reassessed in the light of newer insights into their heterogeneity and function [41–44].





**Figure 6.** Increased apoptosis of CD103<sup>+</sup>CD11b<sup>+</sup> DCs in the absence of a functional SIRP $\alpha$  signal. DC subsets among CD11c<sup>+</sup>MHCII<sup>hi</sup> migratory DCs in whole MLNs isolates from WT and SIRP $\alpha$  mt mice were stained for Annexin V and 7-AAD. (A) Representative plots showing Annexin V and 7-AAD staining by CD103/CD11b DC subsets among CD11c<sup>+</sup>MHCII<sup>hi</sup> migratory DCs. Numbers represent proportions of apoptotic (Annexin V<sup>+</sup> 7-AAD<sup>-</sup>) cells in each subset. (B) Representative plot showing Annexin V and 7-AAD staining in fluorescence minus one controls used to set gates among total migratory (CD11c<sup>+</sup> MHCII<sup>hi</sup>) DCs. (A and B) Plots are from one representative of four experiments, with 3–4 mice/group/experiment. (C) Proportions of apoptotic (Annexin V<sup>+</sup> 7-AAD<sup>-</sup>) cells as a percentage of each DC subset in WT and SIRP $\alpha$  mt MLNs. Data are pooled from four independent experiments, with  $n = 3$ –4 mice/group/experiment. \*\* $p < 0.01$ ; Student's  $t$ -test.

Previous studies have suggested that defective numbers of CD11b<sup>+</sup> DCs in secondary lymphoid organs of SIRP $\alpha$  mt or CD47KO mice might reflect reduced migration via afferent lymphatics [35, 36, 45]. However, this is unlikely to account for our findings, as we observed a similar defect CD103<sup>+</sup>CD11b<sup>+</sup> DCs in both the mucosa and draining MLNs. Although the committed precursors of DCs express SIRP $\alpha$  [31], their numbers were not altered in the BM or blood of SIRP $\alpha$  mt mice. Indeed we found that these pre-DCs actually appeared to be more efficient at generating CD103<sup>+</sup>CD11b<sup>+</sup> DCs in the mucosa compared with WT pre-DCs. This indication that SIRP $\alpha$  may normally function as a checkpoint in the development of a specific subset of intestinal DCs contradicts previous findings in the spleen where pre-DCs from SIRP $\alpha$  mt mice were found to have a reduced ability to generate CD11b<sup>+</sup> DC compared with WT pre-DCs [34]. This could reflect an intrinsic difference in ontogeny between CD11b<sup>+</sup> DCs in systemic lymphoid tissues and intestinal CD103<sup>+</sup>CD11b<sup>+</sup> DCs; indeed the latter DCs

are not found elsewhere in the body. An alternative explanation may be that we analyzed donor cells 5 days after transfer, whereas Saito et al did not examine the fate of pre-DCs in the spleen until 8 days after transfer [34]. Interestingly, these groups also found that the progeny of SIRP $\alpha$  mt pre-DCs had an unusually short half-life in vivo and indeed, we did not find enhanced generation of CD103<sup>+</sup>CD11b<sup>+</sup> by SIRP $\alpha$  mt pre-DCs when the intestine was examined 7 days after transfer. Thus, this earlier study may have missed the accelerated development that we observed.

The idea that CD103<sup>+</sup>CD11b<sup>+</sup> DCs in SIRP $\alpha$  mt mice are compromised in their survival was supported by their increased susceptibility to apoptosis in the MLNs. Although the lengthy enzymatic digestion needed to isolate LP DCs precluded analysis of apoptosis in these cells, all CD103<sup>+</sup>CD11b<sup>+</sup> DCs in the MLNs are within the “migratory” gate, indicating they have come from the mucosa [3, 46]. Thus reduced survival is likely to be an intrinsic property of this subset that resides in LP as part of its life cycle. Whether these cells actually die in the mucosa itself, or their survival defect only becomes apparent once they have migrated to the MLNs remains to be determined.

Overall our results demonstrate a previously unappreciated role for SIRP $\alpha$  in the homeostasis of CD103<sup>+</sup>CD11b<sup>+</sup> DCs in the intestine. We propose that this subset of DCs develops more rapidly in the absence of SIRP $\alpha$  and is then more susceptible to activation and subsequent death. SIRP $\alpha$  may therefore normally act as a brake on these processes via its ability to inhibit signaling pathways by binding SHP1 phosphatase [12]. However, an alternative explanation could come from the finding that SIRP $\alpha$  can also promote survival pathways in other cell types via SHP2-mediated induction of MAPK, PI3 kinase, and NF- $\kappa$ B [47]. Loss of SIRP $\alpha$  signaling could therefore compromise DC survival via this mechanism. As our own and other recent work indicates that SIRP $\alpha$  expressing DCs are also present in substantial numbers in human intestine [6, 10, 30] elucidating the mechanisms of SIRP $\alpha$  mediated control of their development and functions could have important clinical implications.

## Materials and methods

### Mice

Wild-type C57BL/6 (B6) mice were purchased from Harlan Olac (Bicester, UK). SIRP $\alpha$  mt mice [19] were obtained from Dr. PA Oldenberg (Umea University, Sweden) with kind permission from T. Matozaki (University of Tokyo, Japan). CD47KO mice [48] were purchased from Jackson Laboratories (Maine, USA). All strains were backcrossed for at least nine generations on to the B6 background and were maintained under specific pathogen free conditions at the University of Glasgow animal facilities, before being used between 6 and 12 weeks of age. Animal experiments were performed in accordance with UK Home Office guidelines.

## Murine cell isolation

Lamina propria cells were obtained from murine intestines by enzymatic digestion as previously described [3, 49]. Cells were isolated from mesenteric lymph nodes by enzymatic digestion with 1 mg/mL collagenase D (Roche) in calcium magnesium free Hank's balanced salt solution (Gibco, Invitrogen) for 45 minutes. After isolation, cells were passed through a 100  $\mu$ m and then a 40  $\mu$ m filter before use (Corning).

## Flow cytometric analysis and sorting of cells

Cells were stained at 4°C in the dark, as previously described in [49]. For intracellular cytokine staining, whole LP digests were incubated for 4.5 h with 1  $\times$  Cell stimulation cocktail (eBioscience) before fixation and permeabilisation. In all analyses, following doublet exclusion, live cells were identified using 7-AAD (Biolegend) or fixable viability dye (eBioscience). Data were acquired on an LSR II or FACS Aria I (BD Biosciences) and analyzed using FlowJo software (Tree Star Inc).

## T-cell polarization in vitro

Ultra low adherence 24-well plates were coated with 1.5  $\mu$ g/mL anti-CD3 and 1.5  $\mu$ g/mL anti-CD28 (BD Biosciences) in calcium magnesium free PBS for 6 h at 4°C. After washing the plates,  $8 \times 10^5$  FACS-purified naïve CD4<sup>+</sup>CD62L<sup>hi</sup> CD25<sup>−</sup> T cells from MLNs were added in 1 mL complete RPMI supplemented with 10  $\mu$ g/mL anti-IFN- $\gamma$ , 10  $\mu$ g/mL anti-IL-4, 10  $\mu$ g/mL anti-IL-2, 20 ng/mL IL-6, 20 ng/mL IL-23, 20 ng/mL IL-1 $\beta$  (all BD Biosciences), and 2.5 ng/mL recombinant human TGF- $\beta$  (Peprotech). Cells were incubated at 37°C with 5% CO<sub>2</sub> for 4 days and supplemented with 500  $\mu$ L complete RPMI on day 3. On day 4 cells were harvested and cultured with cell stimulation cocktail for 4.5 h at 37°C with 5% CO<sub>2</sub>. The cells were then harvested and stained for intracellular IL-17a and ROR $\gamma$ t.

## DC: T cell cocultures

CD103<sup>−</sup>CD11b<sup>+</sup> DCs ( $3 \times 10^4$ ) were FACS-purified from the SI LP and pulsed with 2 mg/mL OVA for 2 h. Cells were then washed extensively and cocultured for 4 days with  $1 \times 10^5$  CFSE-labeled naïve CD4<sup>+</sup> OVA-specific Transgenic OTII T cells (sorted as CD62L<sup>hi</sup>, CD25<sup>−</sup>). Following coculture, T cells were restimulated for 4.5 h with 1  $\times$  cell stimulation cocktail (eBiosciences) and IL-17a production was assessed as described above.

## Adoptive transfer of pre-DCs

To expand pre-DCs, CD45.1<sup>+</sup> WT or CD45.2<sup>+</sup> SIRP $\alpha$  mt mice were injected with  $2 \times 10^6$  flt3L secreting B16 tumor cells subcutaneously (a kind gift from Dr. Oliver Pabst, Hannover, Germany)

and 10–14 days later, BM was isolated and RBCs lysed (Stem Cell Technologies). Cells were labeled with eFluor450 CellTrace Violet proliferation dye (eBioscience) and pre-DCs were identified as Lin<sup>−</sup> (CD3, CD19, B220, CD49b, MHCII, and CD11b), CD11c<sup>int</sup> SIRP $\alpha$ <sup>int</sup>CD135<sup>+</sup> cells as previously reported [30]. A total of  $3.5 \times 10^5$  FACS sorted pre-DCs were injected into unmanipulated CD45.1<sup>+</sup>/CD45.2<sup>+</sup> recipients in a 50:50 mixture. Five days later recipient mice were examined for donor cells.

## Assessment of apoptosis

Apoptosis was assessed on MLN cells by staining for Annexin V (BD Biosciences) in conjunction with 7-AAD according to the manufacturer's guidelines and analyzed by flow cytometry.

## C. rodentium infection

*C. rodentium* (ATCC 51459) was cultured with aeration in DMEM to log phase (OD<sub>650</sub> = 1.0) before concentration by centrifugation. WT and SIRP $\alpha$  mt mice were inoculated with  $1 \times 10^9$  *C. rodentium* organisms by oral gavage and the level of infection was quantified by colony counts in feces. On day 7, mice were sacrificed and IL-17a producing cells were identified in MLNs following 4.5 h restimulation with 1  $\times$  Cell stimulation cocktail as described above.

## Statistical analysis

Results are presented as means  $\pm$  1 SD unless otherwise stated and groups were compared using a Student's *t*-test, or for multiple groups, a one-way ANOVA followed by a Bonferroni posttest using Prism Software (GraphPad Software, Inc.).

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- Abbreviations:** HAO: heat aggregated OVA · ILC3: type 3 innate lymphoid cell · LP: lamina propria · MP: mononuclear phagocyte · mt: mutant · SI: small intestine · SIRP $\alpha$ : signal regulatory protein  $\alpha$
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